

Genetic Markers in Idiopathic Pulmonary Arterial Hypertension (IPAH)

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ABSTRACT Idiopathic pulmonary arterial hypertension (IPAH) is a rare disorder with abnormally raised pulmonary arterial pressure. In IPAH, the trigger of the endothelial injury may result from oxidative stress, hypoxia, shear stress, inflammation in conjunction with genetic susceptibility. These epigenetic factors may have an influence on cell growth, differentiation and normal homeostatic functions of the endothelium, by altering endothelial permeability, production of growth factors and coagulation factors. Lung inflammation can lead to increased levels of oxidants that may also contribute to the development of IPAH. When the production of ROS exceeds, the capacity of the cell to detoxify them decreases and the resulting oxidative stress may be harmful to the integrity of biological tissue. Since in IPAH there is disruption of pulmonary artery vasculature, the generation of free radicals may further aggravate tissue injury and in view of its role in defensive mechanism, qualitative variation of SOD, CAT, AAT in IPAH is investigated in the present study to correlate specific electromorphic associations in its etiopathogenesis. In conclusion the present study revealed that Oxidative stress pathway (ROS/RNS) and inflammatory components may act as modifiers in pathogenicity of IPAH.

INTRODUCTION

An inflammation in the lung can lead to respiratory burst which is one of the contributory factors in the development of Idiopathic pulmonary arterial hypertension (IPAH) as suggested by Tuder et al. (1994). IPAH is characterized by hypertrophy or proliferation of the smooth muscle in the media of pulmonary arteries initiated by a component of active vasoconstriction (Gaston 1994 and Wink 1996). Apart from the candidate genes, modifiers like endothelial cell derivatives, inhibitors of fibrinolytic pathway, peroxidative products released by the tissue injury, membrane proteins and environmental triggers like oxidative stress may have a role in the progression of the disease.

Oxidant stress enhances the disruption of the mitochondrial electron transport chain, the final effect being either cell injury (necrosis) or apoptosis. Thus, reactive oxygen species (ROS) produced plays a significant role in the

pathogenesis of lung diseases where oxidant stress is increased. The first ROS produced in the reduction pathway of oxygen to water is the superoxide anion, which participates in the generation of other toxic metabolites, most importantly hydrogen peroxide (H_2O_2), hydroxyl radical and peroxynitrite. Hydrogen peroxide, superoxide and hydroxyl radicals and peroxynitrite exert direct cellular toxicity, leading to endothelial dysfunction, proliferation and apoptosis of vascular smooth muscle cells (VSMCs). Superoxide dismutase is an enzyme that exists in 3 isoforms SOD1 (SOD A), SOD 2 (SOD B) and SOD3 (SOD C) which converts reactive superoxide ions to hydrogen peroxide with the latter being detoxified by glutathione peroxidase or catalase subsequently. Studies have suggested that a chronic increase in oxidative stress in the myocardium, possibly due to impairment of SOD and other antioxidant pathways, could contribute to myocardial remodeling and failure (Dhalla 1996 and Hill 1997). Catalase is one of the most potent catalysts known to function synergistically with superoxide dismutase to prevent free radical induced tissue damage. SOD converts the dangerous superoxide radical to hydrogen

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peroxide which is then converted to water and oxygen by the enzyme catalase, a hydroperoxidase. The enzyme is localized to 11p13 that catalyses conversion of hydrogen peroxide, a powerful and potentially harmful oxidizing agent to water and molecular oxygen by disproportionation mechanism. The hydrogen peroxide released promotes oxidization of toxins including phenols, formic acid, formaldehyde and alcohols, performing a peroxidative reaction. Hence Catalase is bifunctional in nature.

In view of their role as antioxidants and their predominant distribution in lungs, the isoenzymic pattern distribution of Superoxide dismutase and catalase was observed in Control and IPA groups.

Human alpha-1 antitrypsin (AAT), is a serine protease inhibitor (serpin) with potent inhibitory activity against neutrophil elastase, matrix metalloproteinases, collagenase in controlling collagen and extra cellular matrix (ECM) protein degradation and appears to be involved in regulation of the immune system. The gene is localized to 14q32.1. In acute inflammatory conditions, increased levels of AAT were observed which prevent the tissue damage induced by proteases during tissue injury (Moraga 2000). The extracellular matrix is remodeled by the process of matrix protein degradation and synthesis triggered by the high blood flow and pressure in the pulmonary vasculature. The serum levels of some of the common phenotypes as a result of single amino acid substitution are: PiMM: 100% (normal), PiMS: 80%, PiSS: 60%, PiMZ: 60%, PiSZ: 40% and PiZZ: 10-15% (severe alpha 1-antitrypsin deficiency). In view of its role on elastase, matrix metalloproteinases of the arteries, the substrates for AAT, this was examined for its polymorphic variation and possible association of specific phenotype with the disease condition.

MATERIALS AND METHODS

54 IPA and equal number of age and sex matched control blood samples were analyzed that were collected from Care and Gandhi Hospitals, Hyderabad, India. The samples were collected along with prior informed consent form approved by Institute of ethical committee. The samples were further processed for the analysis depending on the type of marker studied.

Superoxide Dismutase

Red Cell Membrane Preparation

The whole blood was mixed thoroughly by adding equal amount of saline (0.9% NaCl) and then centrifuged at 2000rpm for 10mins. The supernatant containing the plasma proteins was discarded. Saline was again added to the sample and the above washing step was repeated thrice. Later equal amount of chilled water was added, mixed well and centrifuged at 2000rpm for 10mins. The supernatant was discarded and this step was repeated till a white pellet (red cell membrane) was obtained. The sample was diluted with distilled water and stored at 4°C.

8% Polyacrylamide gel (PAGE) was prepared as per Davies (Davies 1964) protocol, taking tris(hydroxymethyl methylamine), acrylamide-bisacrylamide, ammonium per sulphate solutions and water in the ratio of 1:2:4:1. After the gel was polymerized, a stacking gel of 5% was prepared using tris, acrylamide-bisacrylamide, ammonium persulphate and water in the ratio of 1:2:4:4 and stacked onto the 8% Polyacrylamide gel. Sample of 40ml mixed with 5ml of bromophenol blue dye was loaded and the gel was run at 150volts with constant power supply of 30mA. The staining solution was prepared by taking 5ml of 500mM (K_2HPO_4) Phosphate buffer (pH 7.8), 8.5 ml of 2.8 mM Riboflavin, 10 mg of NBT and 0.220 µl TEMED which is made up to 50ml with distilled water. After the sample was run from cathodic to anodic end, the gel was carefully placed in a staining solution for 45 minutes and was washed with water. The gel was then exposed to fluorescent light for 10mins till the achromatic bands appeared on the blue background of the gel.

Three types of phenotypes were identified based on the mobility as SODA 2-2 (slow moving), SODA 2-1 (medium) and SODA 1-1 (fast moving) respectively.

Catalase

Haemolysate Preparation

An amount of 0.2ml whole blood was taken and washed 3-4 times with 0.9% normal saline to remove the traces of plasma proteins. The sample was frozen, thawed and was resuspended with

60µl of ice cold water to lyse the RBC. The supernatant (haemolysate) was diluted twice its volume for phenotyping of catalase.

7% polyacrylamide gel was prepared by using tris(hydroxymethyl methylamine), acrylamide-bisacrylamide, ammonium per sulphate solutions and water in the ratio of 1:2:4:1 following Davies (1964) protocol. 40µl of the sample (haemolysate) was loaded to the gel and was set up for electrophoretic run. The gel was then stained with specific staining solution prepared by taking equal amounts of Acetate buffer (Equal amounts of 1.5 M acetic acid and 1.5M sodium acetate) and O-dianisidine solution (0.3% in methanol), 0.4 ml of hydrogen peroxide, later made upto 100ml with distilled water before use.

The gel was immersed in the above staining solution and was kept in dark till clear dark brown bands were developed. The slow moving band was identified as HP I and the fast moving band was identified as HP II and presence of both bands indicates Hp I-II phenotype.

Alpha 1 Antitrypsin

7% polyacrylamide gel was prepared as per Davies (1964) protocol with tris, acrylamide-bisacrylamide solution, ammonium per sulphate solution and distilled water in the ratio of 1:2:4:1. 40ml of sample mixed with 5ml bromophenol blue was loaded onto the sample wells. The electrophoresis was carried at a constant current of 20mA at 150volts connected to a DC power supply.

Immunoblotting

The gel was immunoblotted with 60µl of specific AAT antisera (antihuman Alpha-1-antitrypsin, Sigma/grade) diluted in 2ml of saline (0.9%NaCl) which was spread uniformly over the Whatmann (3mm) filter paper of gel size. This filter paper was layered over the gel followed by placement of few moistened filter papers as towels and heavy weight of ~1kg. The whole setup was kept for overnight at 4° C.

The weight and filter paper was removed carefully and the gel was washed thrice with (0.9% NaCl) normal saline to remove any unbound antisera. The gel was then stained with Coomassie blue (25gms of Coomassie blue, 40ml methanol, 7ml of acetic acid in 100ml distilled water) for 30mins followed by destaining. The

gel was destained using destaining solution (7ml of acetic acid and 5ml of methanol in 100ml distilled water) for several times till clear blue bands were visible and was phenotype as per mobility of the bands as F, M, S, Z bands.

The Hardyweinberg equilibrium of the phenotypes was carried out by Chi square test of analysis for possible association of specific electromorphs. And Odds ratio for risk estimates was carried out at 5% level of significance.

RESULTS AND DISCUSSION

Oxidants not only cause cell and DNA toxicity, they even participate in the regulation of cell homeostasis and modulate signaling pathways associated with cell growth and proliferation (Gutteridge 2000). Oxidative stress can be characterized by an imbalance between the generation and the scavenging of ROS and RNS (e.g., O₂⁻ or hydrogen peroxide) and has been identified as a key component in the pathogenesis of central and peripheral diseases (Mates 1999). Pulmonary antioxidant defenses are widely distributed and include both enzymatic and nonenzymatic systems (Heffner 1989 and Halliwell 1996). The major enzymatic antioxidants are superoxide dismutase (SOD), catalase, and the glutathione (GSH) redox system, which inactivates H₂O₂ and hydroperoxides. Since IPAH is a result of endothelial injury of pulmonary artery, the generation of free radicals may further aggravate tissue injury. In view of their role as defensive factors, electrophoretic variation of SOD, CAT, AAT in IPAH is investigated to correlate specific electromorphic associations in its etiopathogenesis. As IPAH is a very rare and fatal disorder, the present study includes only 54 samples reported all over the state of Andhra Pradesh. The Candidate or modifier genes, as the encoded products of these alleles are well established in terms of their properties and function.

Table 1 gives the frequency distribution of SOD, Catalase and Alpha 1 antitrypsin phenotypes. SODA which is Cu-Zn SOD is encoded by two allelic forms SODA1 and SODA2. SODA1 allele encodes for a stable enzyme and SOD A2 allele encodes for unstable enzyme. In the present study the frequency of SOD 1-1, SOD 2-1 and SOD 2-2 phenotypes were found to be 11.11%, 16.66% and 72.22% in controls and 14.81%, 27.77%, 57.4% in IPAH group.

Table 1: Frequency distribution of SOD, Catalase and Alpha 1 antitrypsin in Control and IPAH

Phenotypes		Control		IPAH		χ^2
		n	%	n	%	
Superoxide dismutase	1-1	6	11.11	8	14.81	2.7
	2-1	9	16.66	15	27.77	
	2-2	39	72.22	31	57.40	
Catalase	I	40	74.07	28	51.85	8.41*
	I-II	11	20.37	26	48.14	
	II	3	5.55	-	-	
Alpha 1 antitrypsin	FF	19	35.18	2	3.7	31.38**
	FM	23	42.59	18	33.33	
	FS	2	3.7	2	3.70	
	MM	2	3.7	22	40.74	
	MZ	8	14.81	5	9.25	
	MS	-	-	5	9.25	

*p ≤ 0.05; **p ≤ 0.01

Preponderance of SOD 2-2 (72.22%) in control and SOD 1-1 (14.81%) and SOD 2-1 (27.77%) phenotypes in the disease group was observed, with the variation being statistically insignificant (χ^2 2.7).

Catalases fall into two main classes namely the HP I and HP II catalases. HP II type catalyses the disproportionation of H_2O_2 whereas HP I type has bifunctional activities. It is also known that HP I catalases exist in two isozymic forms, HP I-A and HP I-B respectively. Our study revealed the frequency of Catalase HP I, HP I-II and HP II phenotypes to be 74.07%, 20.37%, 5.55% in the control subjects while 51.85% patients reported to have HP I and 48.14% of HP I-II phenotypes, indicating a predominance of HP I phenotype in the control and HP I-II phenotype in the disease group. The distribution indicates the predominance of heterozygotes in the disease group with a significant association (χ^2 8.41, p ≤ 0.05).

AAT is highly polymorphic and to date, at least 130 unique variant alleles of the AAT gene have been identified that vary in their function and activity (Crystal 1989; DeMeo 2004 and Chappell 2004). Patients exhibiting homozygous AAT deficient alleles are generally considered to be at greater risk, than with a single AAT deficiency allele. Hence, Z and S deficiency alleles account for the various diseases which are a result of single base substitution that substantially reduce the concentration of circulating AAT. The phenotypic distribution of AAT in this study revealed 35.18% of FF, 42.59% of FM, 3.7% of FS, 3.7% of MM, 14.81% of MZ in control group compared to IPAH where 3.7% of FF, 33.33% of FM, 3.7% of FS, 40.74% of MM, 9.25% of MZ and 9.25% of MS phenotypes was

observed. Preponderance of FF, FM, MZ phenotypes in control and MM and MS phenotypes in the disease group was observed. Generally MM phenotypes are found normally distributed in various populations but significant association of this phenotype with the disease (χ^2 3.38, p ≤ 0.05; 0.01) group compared to the control is observed. An association of MM phenotype with the disease reflects the maintenance of homeostasis between aggressive and defensive factors especially in the lung tissue as M codes for a normal enzyme activity. The multifaceted role of AAT as an immunomodulator, acute phase reactant protein and as an antioxidant can justify the association of MM phenotype with the disease. An interesting observation made is that the MS phenotypic individuals are prone to the disease, clearly pinpointing the role of S and M and their encoded products in the etiopathogenesis of IPAH, as S codes for a deficient enzyme. However, to substantiate the finding, larger sample study warranted.

The odds ratio (Table 2) for possible association of SOD, Catalase and Alpha 1 antitrypsin in disease group in comparison to the control group was given in Table 2. In SOD 2 Vs 1 comparison the odds ratio was found to be 3.08 (95% CI: 1.81 -5.2) suggesting the significant association of SOD 2 allele in IPAH. The odds ratio was found to be 0.477 (95% CI: 0.165-1.35) in SOD 2-2 Vs 2-1 comparison, in 2-2 Vs 1-1 the ratio was 0.59 (95% CI: 0.16- 2.1), 2-1 Vs 1-1 comparison gave a risk of 1.25 (95% CI: 0.26 - 5.88) while in 2-2 Vs other comparisons the risk was 0.51 (95% CI: 0.21 -1.2). The Odds ratio of Catalase HP I vs HP II was 0.58 (95% CI: 0.28-1.22), HP I-I vs HP I-II was 0.29 (95% CI: 0.11-

Table 2: Odds ratio for possible association of SOD, Catalase and Alpha 1 antitrypsin phenotypes in IPAH compared to Control group

	<i>Phenotypes</i>	<i>Odds ratio</i>	<i>CI</i>
Superoxide dismutase	2 Vs 1	3.08	1.81 - 5.2
	2-2 Vs 2-1	0.47	0.16 - 1.35
	2-2 Vs 1-1	0.59	0.16 - 2.16
	2-1 Vs 1-1	1.25	0.26 - 5.88
	2-2 Vs 2-1/1-1	0.51	0.21 - 1.2
Catalase	HPI vs HP II	0.58	0.28 - 1.22
	HPI-I vs HPI-II	0.29	0.11 - 0.75
	HPI-I vs HP II-II	-	-
	HPI-I vs HPI-II/HP II-II	0.37	0.15 - 0.91
	HPI-II vs HP II-II	-	-
Alpha 1 antitrypsin	MM Vs FF/ZZ/SS	104.5*	10.53 -1802.4
	FF Vs MM/ZZ/SS	0.01	0.001 - 0.095
	ZZ Vs MM/FF/SS	-	-
	SS Vs FF/MM/ZZ	-	-
	MM Vs M/MZ/MS	12.17	2.14 - 82.62
	FF Vs FM/FZ/FS	0.132	0.01 - 0.70
	ZZ Vs FZ/MZ/SZ	-	-
	SS Vs FS/MS/ZS	-	-

0.75), HP I-I vs HP I-II/HP II-II was 0.37 (95% CI: 0.15 - 0.91). No significant association of catalase phenotypes with IPAH in comparison to the control group was observed though in general, predominance of HP I-II phenotype was observed in IPAH. The confounding effect of the polymorphic/genetic markers clearly reflects the small sample size and the rarity of IPAH in our population due to underdiagnosis and/ or high mortality. Hence, large sample size is warranted to identify such associations of the antioxidants. The possible phenotype at risk is also examined by carrying out the Odds test of Alpha 1 antitrypsin. MM vs FF/ZZ/SS comparison revealed an odds ratio of 104.5 (95% CI: 10.53 - 1802.4), 0.01(95% CI: 0.001-0.095) risk with FF vs MM/ZZ/SS comparisons. The risk was 12.17(95% CI: 2.14-82.62) with MM vs FM/MZ/MS, 0.13 (95% CI: 0.01-0.70) risk in FF vs FM/FZ/FS phenotypic comparisons. Thus, a significant association of wild type 'M' allele with IPAH was observed. The normal levels of AAT protein production was observed in IPAH which can be explained on similar lines of immunomodulation, synergistic action of AAT and NO, acute phase and antiproteolytic enzymes. However, the observation needs to be strengthened by a large sample study.

Table 3 gives the allelic frequencies of SOD alleles and Catalase and AAT alleles in control and disease group. The allelic frequency of SOD2 and SOD1 was reported to be 0.80 and 0.19 in Control and 0.71 and 0.28 in IPAH group

respectively. No variation was found in allelic distribution. There was a significant deviation from Hardy Weinberg expectations in maintaining genetic equilibrium in Control group truly reflecting small sample size. However the preponderance of SOD 1 allele in disease group indicates a stable enzyme production and normal scavenging mechanism emphasizing on the role of other antioxidants like: Vitamin C, vitamin E and β -carotene. However, the synergistic action with of SOD with Catalase and NO cannot be ruled out.

The allelic frequency of HP I and HP II was found to be 0.84 and 0.15 in control group and 0.75 and 0.24 in IPAH, with no deviation from Hardy Weinberg expectations either in Control or IPAH groups, further strengthening our observation of predominance of heterozygotes who are at a selective disadvantage to the disease.

Whereas the allelic frequency of F, M and S was found to be 0.58, 0.32 and 0.09 respectively in control group while in IPAH the allele frequency for F, M, S and Z was 0.22, 0.66, 0.064 and 0.046 respectively. There is an increasing evidence of novel biological effects of AAT beyond its inhibition of elastolysis, such as inhibition of pro-inflammatory responses (Aldonyte 2004). The multifaceted properties of AAT may broaden the AAT role in the lung structural maintenance, its impact in development and in systemic diseases characterized by inflammation, oxidative stress, and apoptosis, as IPAH may involve any one of the pathway in its etiopathogenesis.

Table 3: Allelic frequency of SOD, Catalase and Alpha 1 antitrypsin.

Phenotypes	Phenotypic frequencies						Alleles	Allelic frequencies	
	Control			IPAH				Control	IPAH
	Obs	Exp	χ^2	Obs	Exp	χ^2			
<i>SOD</i>									
2-2	39	34.56	0.57	31	27.22	0.52	2	0.80	0.71
2-1	9	16.2	3.2	15	21.06	1.74			
1-1	6	1.94	8.49	8	4.23	3.36	1	0.19	0.28
χ^2			12.26**			5.62			
<i>Catalase</i>									
HPI	40	45.36	0.63	28	30.37	0.18	I	0.84	0.75
HPI-II	11	13.5	0.46	26	19.44	2.21			
HPH	3	1.21	2.64	-	3.11	-	II	0.15	0.24
χ^2			3.73			2.39			
<i>Alpha 1 antitrypsin</i>									
FF	19	18.16	0.039	2	11.88	8.21	F	0.58	0.22
FM	23	20.04	0.43	18	15.68	0.34	M	0.32	0.66
FS	2	-	-	2	-	-			
MM	2	5.52	2.24	22	35.64	5.22	Z	0	0.064
MS	8	-	-	5	-	-	S	0.09	0.046
MZ	-	-	-	5	-	-			
χ^2			2.72			13.78			

*p ≤ 0.05; **p ≤ 0.01

Table 4: Frequency distribution of Superoxide dismutase phenotypes with respect to genetic factors in Control and IPAH.

Genetic markers	SOD Phenotypes						χ^2	
	1-1		2-1		2-2			
	C	IPAH	C	IPAH	C	IPAH		
Catalase								
HPI	10	10.7	17.5	28.57	72.5	60.7	27.64**	0.74
HPI-II	18.1	19.2	81.8	26.9	-	53.8		
HPII	66.6	-	33.3	-	-	-		
χ^2		3.42		1.19		17.08**		
AAT								
FF	5.2	-	21	50	73.6	50	11.33	6.82
FM	17.3	16.6	21.7	38.8	60.8	44.4		
FS	50	-	50	50	-	50		
MM	-	18.1	-	13.6	100	68.1		
MS	-	20	-	40	-	40		
MZ	-	-	-	20	100	80		
χ^2		7		7.43		26.74**		

*p ≤ 0.05; **p ≤ 0.01

Frequency distribution of Superoxide dismutase phenotypes with respect to genetic factors in control and IPAH is presented in Table 4. The difference was found to be significant with respect to catalase phenotypes and SOD 2-2 (χ^2 17.08; p ≤ 0.05; 0.01) phenotypic comparisons, clearly depicting synergism between catalase and SOD phenotypes on intergroup comparisons.

Intergroup comparisons with respect to Alpha-1-antitrypsin phenotypes, indicate that, AAT seems to be significantly influencing SOD 2-2 phenotype (χ^2 26.74; p ≤ 0.05; 0.01)

suggesting the pleiotrophic action of AAT and SOD. Thus the above finding clearly reveals interactive role of catalase and AAT with SOD.

CONCLUSION

The present study revealed that Oxidative stress pathway (ROS/RNS) and inflammatory components may act as modifiers in the disease pathogenicity. A significant association of SOD 2-2 phenotype with AAT and Catalase phenotypes observed indicates that AAT influences

SOD and also reveals interactive role of catalase and AAT with SOD in scavenging the reactive oxygen species or in the progression of the disease as modifier gene effects.

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